THE MANNER OF SPERM ENTRY IN THE STARFISH EGG

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In an article published several years ago (Chambers, 1923) I described some morphological aspects of the insemination of the starfish egg. A peculiar feature in this process, the interpretation of which has been adversely criticized (Lillie and Just, 1924; Just, 1929) is the apparently passive and relatively slow travel of the blunt-headed spermatozoön through the jelly which surrounds the egg.

There is a striking contrast between the arrangement of the spermatozoa about freshly inseminated starfish (*Asterias*) and sea-urchin (*Arbacia*) eggs. In *Arbacia* the pointed, narrow-headed sperm quickly pass through the jelly surrounding the eggs and, within a few seconds after insemination, are on the surface of the egg. In *Asterias* the blunt, ovoid sperm penetrate very little into the jelly and collect on its outer border far from the surface of the eggs. By careful observation, one is able to detect a spermatozoön, advancing through the jelly by a peculiar gliding movement to the egg. As described in my previous paper, the moment when the spermatozoön starts to migrate through the jelly, it is seen to be connected by a tenuous filament to a conical elevation on the surface of the egg. The spermatozoön advances as the filament progressively shortens until the head of the spermatozoön finally reaches the cone into which it sinks. From there it travels into the main body of the egg.

Fol, who was among the first to describe the penetration of a spermatozoön into an animal ovum (Fol, 1877) made an extensive study of the process in *Asterias* and *Toxopneustes* (Fol, 1879). In his studies on the starfish he was struck by the peculiar directive movement of the spermatozoön through the jelly to a conical elevation on the surface of the egg and considered the possibility that the progress was due to the retraction of a filament, connecting the spermatozoön with the cone, Fig. 1. He dismissed the idea that the filament is an outgrowth of the spermatozoön, since he observed no diminution in volume of the head. He also suggested that protoplasmic filaments may pre-exist extending from the egg through the jelly and that a sperm, coming into contact with one of these filaments, may be drawn in by a reaction on the part of the egg. Not being able to observe such a filament except as a comparatively short extension of the cone, Fol concluded that the initial

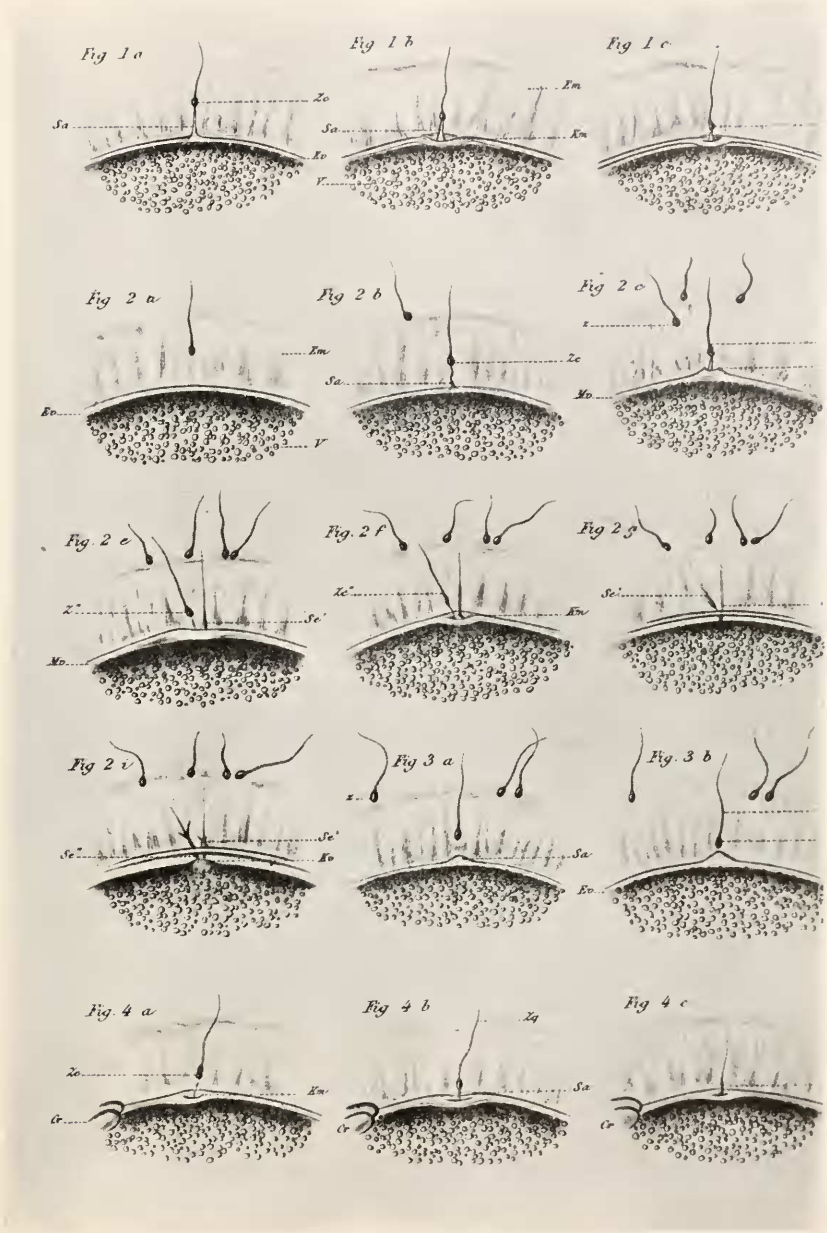

FIG. 1. Photographic reproduction of part of Plate III from Fol's paper (1879) on *Asterias glacialis*, the drawings of which were from the living egg. In Fig. 1, *a*, *b*, and *c* are three successive phases of the same zoösperm, *zc*. An extension of the entrance cone is at *Sa*. The phase in which the zoösperm entered is omitted here. In Fig. 2, *a*, *b*, *c* (*d* omitted) *e*, *f*, *g*, (*h* omitted) and *i* are seven views of the same objects; in Fig. 2, *b*, *c*, a zoösperm, *zc*, is approaching. In *e* and *f* a second zoösperm, *z''*, is approaching. In *g* and *i* are extensions, *Sc'* and *Sc''*, of the "cone d'exudation." Fig. 3, *a*, *b*, shows the approach of a zoösperm to an exceptionally large cone. Fig. 4, *a*, *b*, *c*, shows a zoösperm entering near region of polar bodies, *Cr*.

travel of the sperm is due to an attraction exerted by the cone from a distance. A photographic reproduction of a part of Fol's illustrations is shown in Fig. 1.

The results presented in this paper constitute a critical re-examination of the phenomenon and are based upon observations made at different periods every summer since the publication of my original article.

During the summer of 1929 at the Marine Biological Station, Roscoff, France, I was able to confirm the observations of Fol on the species he used, *Asterias glacialis*. Fol's article is remarkable for its wealth of detailed description and should be referred to by any one interested in the subject.

Methods and Material

Observations were made at Woods Hole on the ova of *Asterias rubens*, the common starfish, during all the summer months from June to September. The ova were obtained both by allowing a ripe female to shed the eggs naturally in sea-water and also by removing and cutting up ripe ovaries in bowls of sea-water.

The insemination process was observed with a 3 mm. apochromatic objective in both immature and mature ova at various times before, during and after completed polar-body formation. The temperature of the water in which the inseminations were made varied at different times of the summer (from 15° C. to 20° C.). A preliminary insemination of a sample lot of the eggs was always made under the conditions of the final experiments and only those kept for a study of the normal process when normal fertilization membranes developed within a few minutes on a minimum of 90 per cent of the eggs. For the crucial experiments precautions were taken to make adequate dilutions of the sperm-suspensions in order to procure maximum fertilization with a minimum of sperm present. Heavily inseminated specimens were also studied.

In all the cases in which the penetration of the spermatozoön was observed, the manner of its entry proved to be essentially the same irrespective of variations in temperature, age of eggs or amount of sperm present.

Fol used the following excellent method for observing insemination. He placed a drop of sperm-suspension on the slide of a compressorium on the stage of the microscope and a hanging drop of sea-water containing the eggs on the coverslip of the cap of the compressorium, which was inverted over the slide. After bringing the sperm-suspension into the field of the microscope, he carefully lowered the cap of the compressor until the two drops touched. The eggs, being heavier than the water in which they were suspended, fell through the liquid, while the

sperm rose and encountered the eggs under conditions approaching the normal.

The compressorium used by Fol may be dispensed with if a coverslip be mounted on feet of soft clay and the two drops brought together by pressing down on the coverslip. Owing to the fact that the starfish eggs react relatively slowly (15–45 seconds), the sperm can also be mixed with the eggs in a dish. A drop of the mixture is then placed on a slide and covered for observation. With a little practice one is able to bring the eggs into view under an oil immersion objective within 5–10 seconds. Some of my studies were made with the use of the micromanipulator, the sperm-suspension being microinjected into a hanging drop containing the eggs already under view in the microscopic field. With this method the entire sequence of events could be observed from the moment that the sperm arrived in the vicinity of the eggs.

Experiments were also made in which the microneedle was used to operate on the surface of the egg and to seize entering spermatozoa. For this purpose it was essential to have two observers using a demonstration ocular, one observer maintaining the spermatozoön in focus, while the other observer operated the microneedles. I wish to take this opportunity of expressing my appreciation to Dr. G. H. Faulkner of the University of London, who was of the greatest assistance to me in this way.

The time relations of the several steps in the penetration of the spermatozoön vary within certain limits. Spermatozoa taken directly from the testis are sluggish and frequently motionless, but become active when diluted in sea-water. As long as they are actively motile, the spermatozoa of different batches seem to be similar in their behavior toward eggs of one lot. On the other hand, with eggs of different lots and ages, considerable time-variations occur, although the consecutive steps of the insemination process are the same. Immature eggs, as well as eggs which have maturated and have stood for hours in sea-water can be readily inseminated.

In immature eggs the penetration of a spermatozoön does not always cause the vitelline membrane to rise so as to form the fertilization membrane and, if plenty of sperm be present, the sperm will keep on penetrating until the egg is fairly riddled with them. Polyspermy is also the rule for mature eggs aged for three to five hours.

In freshly maturated eggs the peculiar reaction which prevents polyspermy occurs within an average time of 45 seconds and the fertilization membrane rises rapidly. In some batches of eggs the time

limit of sperm-penetration may be only 75 seconds, although the usual limit is two minutes.

Experimental

A. Observational Studies

1. The Jelly Around the Starfish Egg

The clear jelly which surrounds the egg swells in sea-water to form a layer approximately ⅙ the diameter of the egg. The outer border of this jelly can be shown by the well-known method of placing the eggs in sea-water containing a suspension of india ink. In accordance with Fol's findings, the jelly appears to be principally a matting of delicate fibrillæ arranged in radial lines. Its density is greatest close to the egg and progressively loosens on approaching its external border. Fol used an ingenious method to demonstrate the radial structure by placing eggs in sea-water containing rod-shaped bacteria. The bacteria implanted themselves in the jelly and always in lines perpendicular to the egg's surface.

In the immature condition the jelly is bounded externally by a thin cellular membrane which breaks up as the jelly swells in the water. When this membrane is present the spermatozoa do not adhere to it. As soon, however, as the membrane disrupts, the spermatozoa readily accumulate in the peripheral meshes of the exposed jelly.

The density of the jelly is such that the starfish spermatozoa with their blunt heads remain entrapped in its outermost zone while their tails continually lash to and fro. On the other hand, the narrow-headed sand-dollar and sea-urchin sperm can work their way quite through the jelly of the starfish egg. Their progress is somewhat impeded the farther they penetrate, but they arrive at the surface of the egg within one or two minutes. This is in striking contrast to the few seconds which it takes them to go through the looser jelly of both sand-dollar and sea-urchin eggs.

The jelly of the starfish egg cannot be removed entirely by mechanically shaking the eggs, although such a procedure is frequently successful for sea-urchin and sand-dollar eggs.

2. Insemination of the Freshly Maturated Egg

In an inseminated preparation of eggs in sea-water a microscopic examination will show the spermatozoa adhering to the outer borders of the sticky egg-jelly. As long as the spermatozoa do not touch the jelly they are as likely to swim away from the egg as towards it.

Fig. 2 (*A–Q*) represents seventeen successive steps in the passage

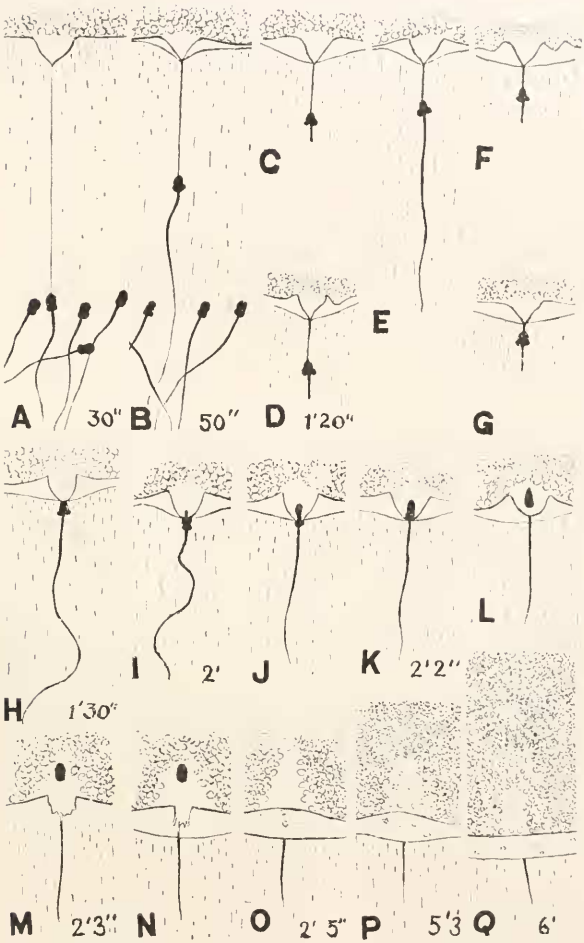

FIG. 2. Seventeen successive steps in the insemination of a starfish egg. For description see text.

of a spermatozoön through the egg-jelly and into the egg until the diminutive sperm-aster becomes appreciable. The drawings were made mostly from observations on one specimen obtained from freshly matured eggs shed naturally in a tank and inseminated with a minimum dilution of spermatozoa to ensure proper insemination. The preparation was brought under observation (with a 3 mm. apochr. objective) within 10 seconds after mixing the eggs with the spermatozoa.

In Fig. 2, *A* several spermatozoa are shown in the outer border of the jelly. When first observed, the head of one of these was already connected by means of a distinctly appreciable but tenuous filament extending through the jelly to a hyaline, conical papilla on the surface of the egg.[1]

Twenty seconds later the spermatozoön had moved about halfway in, Fig. 2, *B*. Its progress was steady and in a straight line, while the tail stretched out motionless behind and only occasionally gave a spasmodic twitch. The fertilization membrane was already to be seen beginning to rise from the cone at the base of the filament. The successive steps in the advance of the spermatozoön to the summit of the cone (Fol's cone d'attraction) are shown in *C* to *H*. While this was occurring, wave-like quivers (see *D* to *G*) passed over the cone and the adjacent surface of the egg. When the spermatozoön reached the summit of the cone, there was an appreciable pause of 30 seconds, after which the sperm-head narrowed at its tip and lengthened out as it slipped through the fertilization membrane to round out again after it has passed into the underlying cone (*I* to *K*). The changes in the shape of the head of the spermatozoön suggest the existence of a pore in the rising membrane through which the filament had previously extended and which is now the means of ingress for the spermatozoön. When once the spermatozoön started to enter, it slipped through rapidly and, within 2–3 seconds, passed definitely into the egg, where its progress (*N–O*) could be followed along an ever-deepening, hyaline pathway caused by a recession of the cytoplasmic granules. As the sperm-head advanced in the egg it became increasingly difficult to see. Within 6 minutes after insemination, the diminutive sperm-aster (*P* and *Q*), became evident at the bottom of the pathway. The path gradually disappeared as granules moved back into it. Usually it is visible for 8 to 10 minutes after insemination.

During the progress of the spermatozoön through the jelly the sperm-tail is relatively inactive. Frequently a spermatozoön moves all the way to the insemination cone without a single twitch of its tail. A

[1] Some of the best observations I have made of this phase were with dark-ground illumination.

pronounced lashing of the tail occurs only during the pause after the spermatozoön has reached the cone, Fig. 2, *H*, and while it is passing through the fertilization membrane, Fig. 2, *I–F*. As long as there is a continuity between the tail and the advancing head within the egg, the tail keeps on feebly lashing. When the connection with the sperm-head is lost, the tail becomes motionless, but can be recognized for a long time (ten to fifteen minutes), extending outward from the fertilization membrane, Fig. 2, *N–Q*.

The fertilization membrane usually becomes evident in the region

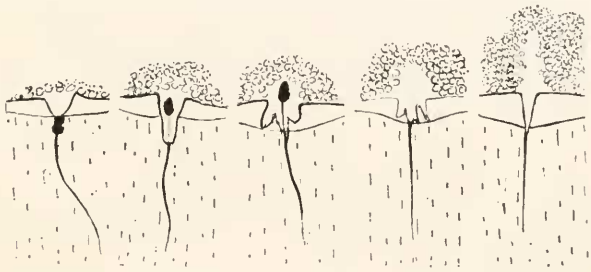

Fig. 3. Progressive changes in the form of an exudation cone.

of the cone before the spermatozoön has migrated halfway through the jelly, Fig. 2, *B*. Its complete elevation over the egg occurs within 5 to 20 seconds later.

The conversion of the entrance cone into the exudation cone (Fol's cone d'exudation) takes place after the spermatozoön has passed into the egg. Ever-changing, flame-like processes develop on the cone, Fig. 2, *M, N*, which finally withdraw and the cone disappears, frequently leaving behind minute globules, Fig. 2, *O–Q*, which become dispersed in the space between the fertilization membrane and the egg. A variation of the exudation cone is shown in Fig. 3.

In over-inseminated eggs several spermatozoa may become attached, Fig. 4, *A*, each to the tip of a filament extending from the egg. Al-

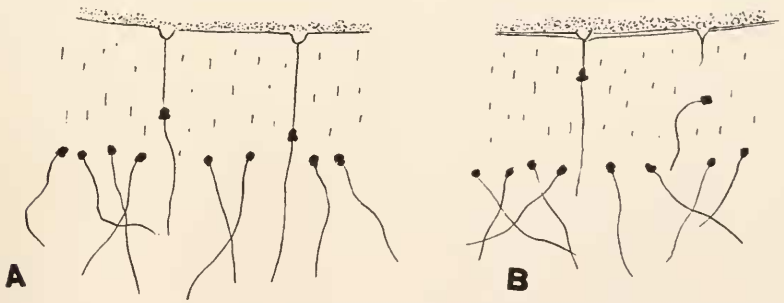

Fig. 4, *A*. Two spermatozoa migrating together into an over-inseminated egg. *B*. One lost its attachment and was discarded, while the other successfully entered the egg.

though these spermatozoa begin to move through the jelly, there is a tendency for only the most advanced one to reach and penetrate the egg. The others, before reaching the egg, tend at one time or another to lose connection with their filaments. Such released spermatozoa, after a spasmodic twitch or two, remain permanently motionless, Fig. 3, *B*, in the jelly. The filaments which have lost their spermatozoa are quickly withdrawn and, together with their cones, soon sink into the egg.

The filaments, extending from a cone to a spermatozoön, are usually at right angles to the egg's surface. That this is not always the case is shown in Fig. 5, where two convergent filaments are shown. This argues against the pre-existence of definite radial canals in the egg-jelly through which the spermatozoa might be supposed to move.

The shape of the head of the spermatozoön, as already commented upon by Fol, occasionally changes considerably as the head moves through the jelly. The change seems to be due mainly to a bulging of the neck-piece on one or both sides of the head, Fig. 6, *A, B* (*cf.* Fig.

Fig. 5

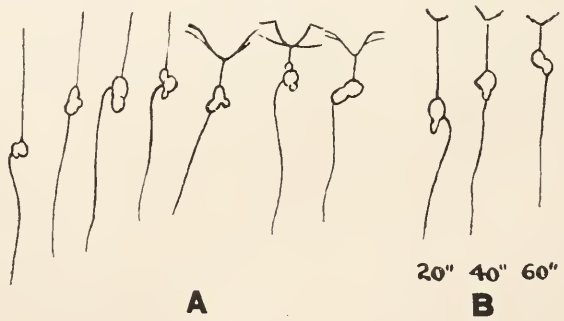

Fig. 6

Fig. 5. Two spermatozoa attached to insemination filaments which are convergent and not radial as usual.

Fig. 6. *A*. Sketches to show variations in shape of the heads of spermatozoa migrating through the egg-jelly. *B*. Changes in shape of the head of one spermatozoön at intervals of 20, 40, and 60 seconds.

5). In Fig. 6, *B* are three sketches of a single spermatozoön, at intervals of 20, 40 and 60 seconds after insemination. The impression that the head of the spermatozoön is bent to one side may be due to the distorted shape of the neck-piece. Occasionally, a spermatozoön appears to be carried through the jelly with the base of its tail at right angles to the attachment of the insemination filament, while the rest of the tail is curved so as to trail behind.

Figures 7–10 represent variations. Fig. 7 shows a sperm-head which was unusual in performing active, wriggling movements for fully one minute after having penetrated the egg while the tail hung motionless outside. During these movements the sperm-head left the usual hyaline pathway and could be seen jostling and pushing aside the cytoplasmic granules encountered.

Fig. 8 shows a spermatozoön whose head, after passing through

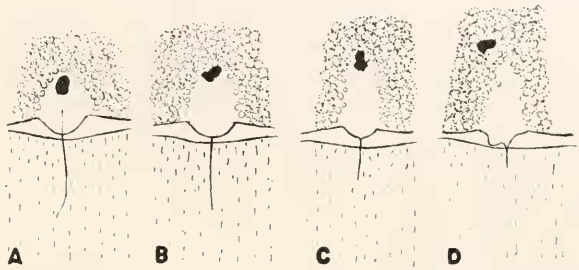

Fig. 7

Fig. 7. Four successive steps in the progress of an unusually active sperm-head after it had penetrated an egg.

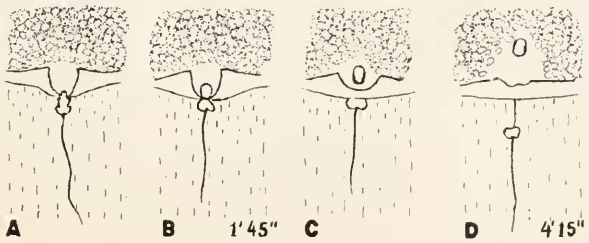

Fig. 8

Fig. 8. A spermatozoön which on entering an egg left its neck-piece outside the fertilization membrane.

the fertilization membrane, broke away from its neck-piece which was left outside with the tail.

Figs. 9 and 10 show the reactions of late arriving spermatozoa. Fig. 9 shows a spermatozoön which succeeded in passing through an already lifted fertilization membrane. During the process the cone changed shape and flattened out, while the fertilization membrane became appreciably indented. In Fig. 10 the spermatozoön reached the cone, *A–C,* but failed to enter. The fertilization membrane wrinkled and the cone formed accessory elevations, *D–F,* but, when the cone finally withdrew from the membrane, the spermatozoön was left out-

side. The head of the spermatozoön then sprang back for a short distance where it remained motionless and attached to the membrane by a slender thread, *G*, nine minutes after insemination.

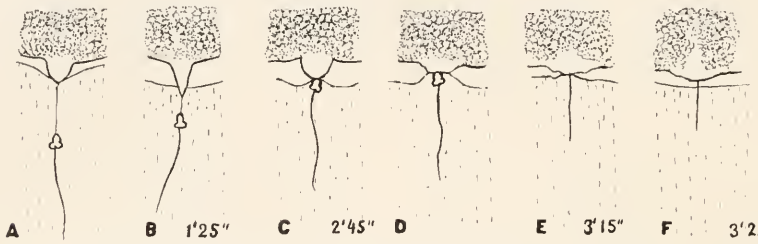

Fig. 9. Delayed entry of a spermatozoön through a fertilization membrane formed by the penetration into the egg of another spermatozoön not shown in the figure.

3. The Origin of the Insemination Filament

The insemination filament is so fine that it is practically invisible except when the cone at one end and the sperm-head at the other end are brought simultaneously into focus. Considerable practice is required to detect the sperm at the moment when it is beginning to migrate into the jelly. In the outer border of the jelly among several spermatozoa whose heads are moving to and fro while their tails lash about, one's attention becomes attracted to a sperm-head which has ceased its side-to-side movements and, instead, is moving steadily and in a straight line into the depths of the jelly. By looking along the direction of its movement, a cone on the egg's surface becomes apparent and, between the cone and the sperm, is to be seen the delicate, tenuous insemination filament. In fresh maturing eggs I have never been able to see the cone without also seeing the advancing sperm and the filament connecting the two. The formation of the filament is apparently too rapid. In immature eggs the cone is relatively much larger and as already described (Chambers, 1923) I have several times observed a tapering extension grow out from it until contact is made with a sperm, whereupon the extending portion retracts and draws the sperm in with it.

In mature eggs which have been standing in sea-water for 2 to 4 hours there is frequently a greater response to multiple cone formation than in fresh, maturing eggs and consequently the chances are better to catch the initial stages. Eggs, 3 hours old, were placed in a shallow hanging drop in a moist chamber and, after being brought under observation, a suspension of sperm was blown into one side of the field by means of a micro-pipette. The spermatozoa quickly spread in the interstices between the eggs and several became attached to the

outer border of the jelly of the egg in view. Within 10 seconds a number of minute, conical, blister-like elevations developed on the egg's surface opposite the sperm. A delicate membrane appeared as if it were being lifted off the egg's surface by the rising cones. A few of the hyaline cones increased in size and, during the several succeeding seconds, there was no sign of any connection between them and the sperm lying on the periphery of the jelly. One cone increased ap-

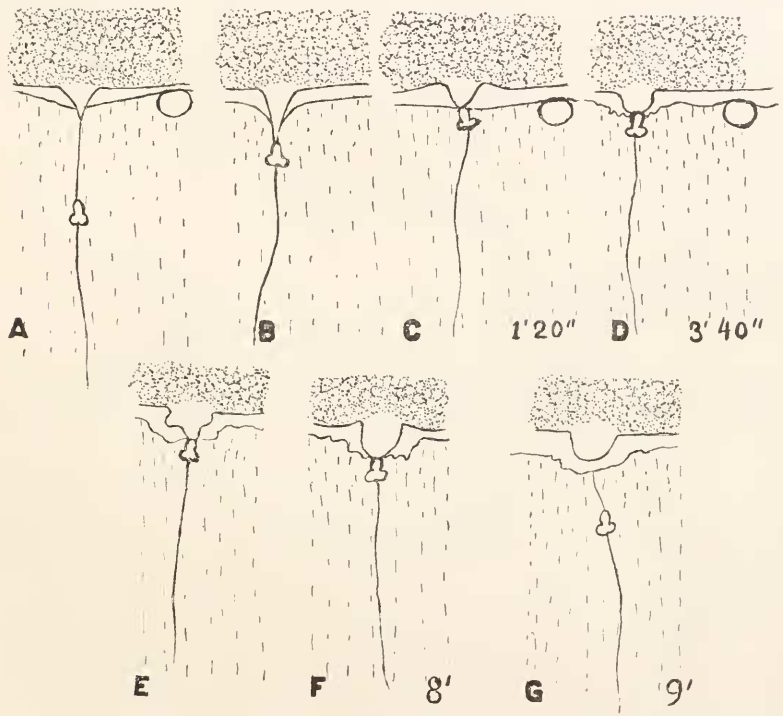

FIG. 10. Attempted penetration of a delayed spermatozoön which was finally discarded.

preciably in size and suddenly, within an instant, a distinct line could be seen connecting its tip with the head of a spermatozoön. The other spermatozoa remained on the surface of the jelly while the spermatozoön in question began to migrate inward. While this was occurring, the rounded surface of the cone tapered more and more and the ever-shortening filament became appreciably thicker.

A curious phenomenon which may be of significance is the fact that, in the majority of cases, the insemination filament always connects with a spermatozoön. Because of this one is almost inclined to believe in

a specific attraction such as Fol suggested. I may cite, for example, a case in which about 30–50 spermatozoa were blown on the surface of an egg. Most of the spermatozoa immediately became attached to a restricted region on the outer border of the jelly. One, however, wandered off a short distance and suddenly a cone appeared with a

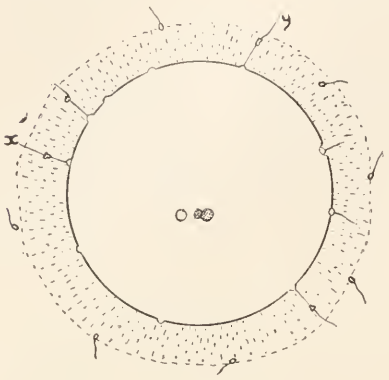

Fig. 11

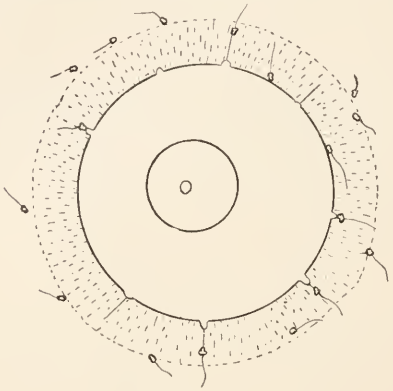

Fig. 12

Fig. 11. Polyspermy in an egg 5 hours old. The egg nucleus and two polar bodies show prominently in the middle of the figure. Sperm at *x*, although more advanced, entered later than sperm at *y*.

Fig. 12. Polyspermy in an immature egg.

tenuous filament extending to the spermatozoön diagonally through the jelly. The filament then retracted with the spermatozoön on its tip and insemination resulted.

4. Insemination of Immature and of Aged Eggs

Eggs aged by standing in sea-water lose their protective reaction against polyspermy. Fig. 11 represents an egg which was inseminated after it had been standing in sea-water for five hours, which is over four hours longer than is usual for normal fertilization. Within one minute numerous cones formed on the egg. The figure shows the egg with six attached spermatozoa, all of which were taken in. Owing to the rapidity of the procedure and the variations in the angles of direction which the filaments take, it was impossible to ascertain whether or not the cones in the figure which show no filaments did in reality possess filaments with spermatozoa attached to them.

There is often a lack of uniformity in the sequence of the sperm entries. In Fig. 11 the spermatozoön at *x* was in advance of its neighbor at *y*. In spite of this, spermatozoön *y* entered before *x*.

One egg, two hours after maturation, formed two cones with in-

semination filaments at an interval of two minutes. Both successfully drew in their spermatozoa. One minute later another cone and filament developed. Its spermatozoön began to be drawn in, but the rising fertilization membrane had appreciably formed and the spermatozoön was discarded.

Another egg, 5 hours old, formed a large number of cones so close together that, as they enlarged, they became more or less confluent and spermatozoa kept migrating into them in large numbers, Fig. 13.

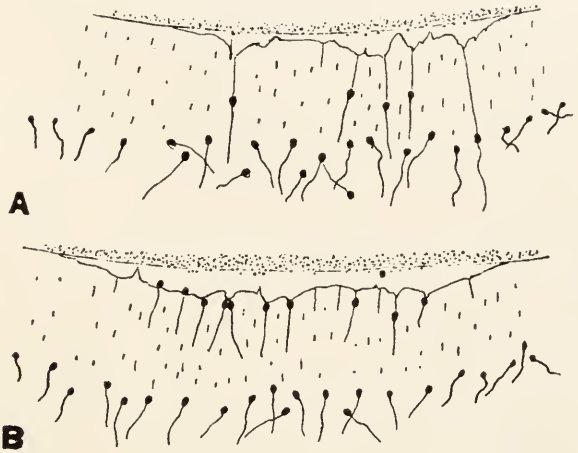

FIG. 13. Excessive over-insemination with formation of confluent cones.

The lack of a protective reaction against polyspermy in old, mature eggs obtains also for immature eggs. This is shown in Fig. 12. The entrance cones which form on the immature egg are distinctly larger than those of the mature egg.

As the sperm passes into an immature egg no hyaline pathway is formed such as occurs in the mature egg. The spermatozoön is quickly lost to view among the cytoplasmic granules and no aster ever develops. Also the exudation cone which forms at the site of the disappearing entrance cone usually develops into a strikingly large prominence with elongated flame-like processes. A membrane similar to the fertilization membrane of mature eggs forms about an immature egg upon insemination. In fresh, immature eggs the membrane seldom rises. It simply toughens as can be demonstrated by the microneedle. In old eggs, which remain immature by maintaining an intact germinal vesicle, the membrane frequently lifts off upon insemination.

5. Time Relationships in the Insemination Process

The time relations of events in the insemination process are shown in the accompanying table, in which records are given on observations of a number of individual eggs.

After the eggs and sperm are mixed there is always an appreciable time of 20 to 35 seconds before the first spermatozoön begins definitely to migrate into the jelly. The average time to pass through the jelly is 60 seconds. The spermatozoön remains on the surface of the entrance cone for about 25 seconds, after which it rapidly penetrates the cone and passes into the interior of the egg. The diminutive sperm-aster becomes appreciable within 5 to 6 minutes after insemination. Within certain limits the sequence of events for fresh, maturing eggs is fairly uniform. The greater variability in old eggs may be due to the fact that aging eggs permit polyspermy and hence the data probably include records on the penetration of late as well as early arrivals.

A comparison of my data with those recently published by Just (1929) and included in the table shows agreement in one essential point, *i.e.*, in the average time taken after insemination for the sperm to enter the cone, viz., 120 seconds. The disagreement lies in the time taken for the sperm to arrive on the cone. Although Just states that he made his observations both on living and fixed eggs, careful perusal of his paper suggests that he depended more on data obtained from fixed and sectioned material than from observations on the living egg. According to my observations, the spermatozoa were never observed to reach the surface of the egg in less than 45 seconds. I cannot explain Just's statement that this occurs within 5 seconds except on the assumption that throwing the eggs into a fixative might possibly induce a sudden contraction of materials so as to bring the sperm on the cone before the fixing agent had time to exert its preservative action.

B. Microdissection Studies

6. Physical Properties of the Cone and of the Insemination Filament

The entrance cone possesses a surprising stiffness somewhat at variance with the impression it gives to the eye from its ever-changing contour.

A cone, Fig. 14, *A*, into which a spermatozoön had just entered, was pushed inwards by means of the tip of a microneedle bearing down on the fertilization membrane, *B*. The relative stiffness of the cone was indicated by the fact that the general contour of the egg about the cone was carried in while the cone persisted in its original form within the

TABLE

Time Relations in the Process of Insemination in Asterias rubens

	Condition of Eggs	Duration of Process	Sperm Added	Spermatozoön				Fertilization Membrane	
				On Filament		Arrived on Cone	Within Cone	Started	Completed
				At Periphery of Jelly	Half Way through Jelly				
My Data	Polar bodies being formed	Longest recorded	0″	45″	60″	100″	135″	45″	60″
		Shortest recorded	0″	15″	30″	55″	80″	30″	35″
		Average (20 observations)	0″	30″	50″	90″	115″	40″	50″
	Aged		0″	variable		60–180″	90–210″		
	Immature		0″	variable		60–180″	120–240″		
Just's Data	Both fresh and fixed		0″			5″	120″	45″	

resulting recess. The microneedle was then passed through the fertilization membrane and the surface of the rounded cone was seized and deformed by pulling, *C*. After removal of the needle, the dragged-out part of the cone slowly and gradually withdrew, *D–E*.

In another case the fertilization membrane was first removed by tearing and the cone pulled out into a long tapering strand. While held

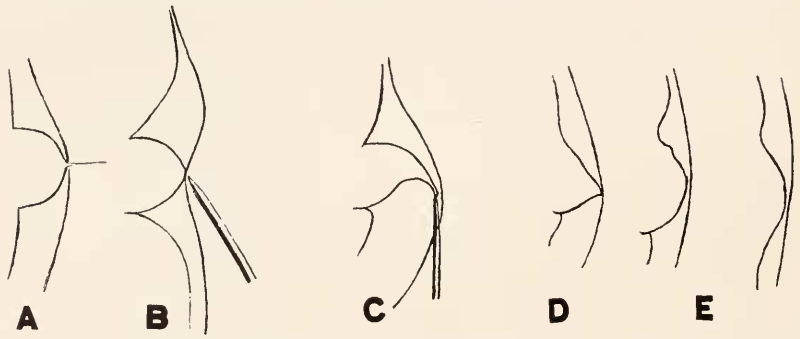

Fig. 14. Micromanipulation of an entrance cone. For description see text.

in this position, the strand became lumpy as if it were breaking into beads. The contour of the cone at its base kept changing, while the lumpiness of the strand progressively disappeared and reappeared. Finally the strand broke into beads. The basal position of the strand, thus freed from the needle, gradually sank into the cone, which ultimately flattened out and disappeared. This phenomenon is similar

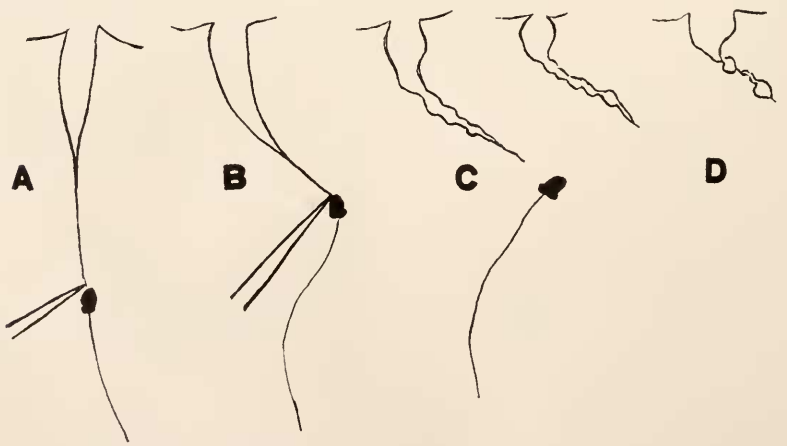

Fig. 15. Effect of removing spermatozoön from the insemination filament.

to what occasionally occurs when the lifting of a fertilization membrane, due to insemination elsewhere on the egg, drags out a retracting filament attached to a spermatozoön outside the membrane.

With a microneedle a spermatozoön was removed from its filament while the sperm was moving through the jelly, Fig. 15. The tip of the needle was raised and moved against the spermatozoön, *A*. In the process the cone became stretched as the filament was pushed to one side, *B*. Eventually the spermatozoön became dislodged, *C*, whereupon the filament retracted and beaded, *D*, while the freed spermatozoön remained motionless in the jelly. In other cases I have tried without success to separate the filament from its cone by manipulating the needle-tip where the filament joins the cone. The filament continues retracting and the spermatozoön moves steadily to the cone except when the operation becomes so brutal as to disrupt the cone.

7. The Effect of Removing the Vitelline Membrane before Insemination

I have already described the fertilization of eggs previously deprived of their vitelline membranes, (Chambers, 1923). The jelly adheres to the membrane which in its turn is closely adherent to the egg. While tearing the membrane the egg is usually injured. Occasionally, however, one is able to insert a fine needle under the membrane, Fig. 16, and lift it off while delicate strands of protoplasm which appear, stretch and break. The following experiment indicates that this membrane is the same structure which lifts off as the fertilization membrane. The

Fig. 16

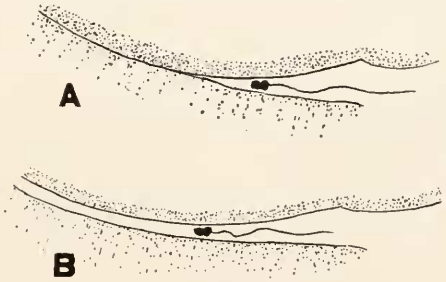

Fig. 17

Fig. 16. Operation of tearing the vitelline membrane of an unfertilized egg.
Fig. 17. An egg inseminated after partially tearing off the vitelline membrane. *A*. Spermatozoön lying in space between vitelline membrane and egg. *B*. Fertilization membrane lifted owing to insemination by a spermatozoön not shown in figure.

membrane was partially torn from the surface of an egg which was then inseminated, Fig. 17. A spermatozoön happened to find its way into the space under the torn membrane, *A*, while the egg was fertilized by another spermatozoön in a region not shown in the figure. The lifting of the fertilization membrane spread over the egg until it reached the torn region, where the presence of the horizontally stationed spermatozoön

showed that the fertilization membrane was identical with the membrane which previously had been torn, *B*. The spermatozoön in the figure advanced somewhat within the space between the egg and the membrane.

Fig. 18 shows the way in which the jelly can be removed from an unfertilized mature egg. After tearing the jelly, the exposed part of the egg is seized with one needle while the jelly at the other end of

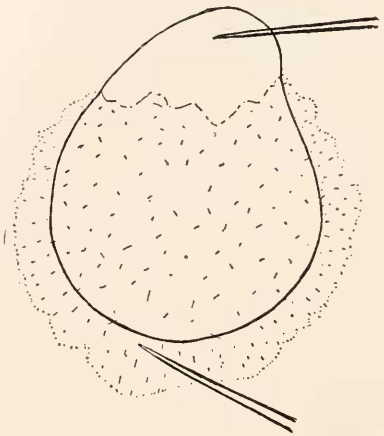

Fig. 18. Method of removing an egg from its investing vitelline membrane jelly.

the egg is caught by a second needle. By gentle manipulation, the egg can be drawn completely out of its jelly. Such an egg at the outset is very sticky. However, by rolling it about, the adhesiveness diminishes and the egg rounds up and cannot be distinguished from untreated eggs except for the lack of an investing jelly.

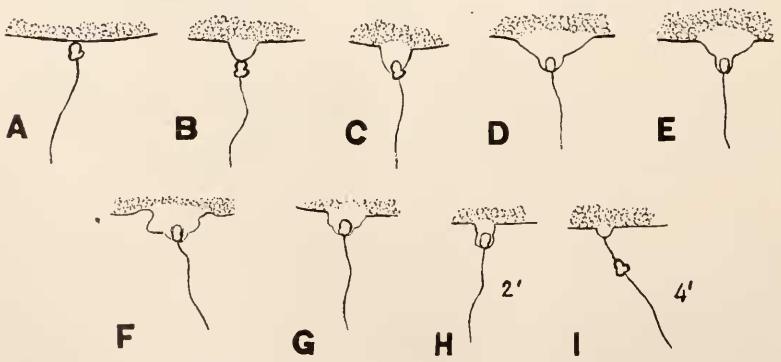

Fig. 19. Several steps in an unsuccessful insemination of a naked egg.

Fig. 19 represents an unsuccessful attempt at fertilizing a naked starfish egg. An entrance cone developed at the spot where a spermatozoön touched it, *A, B*. The head of the spermatozoön was engulfed by the cone, *C* and *D*. However, the sperm-head did not move inward, *E*. Instead, the cone spread out at its base, became irregular, *F*, and then diminished in size, *G* and *H*. Finally the spermatozoön was expelled, *I*, four minutes after it had arrived on the surface of the egg.

Fig. 20 represents the stages of a successful sperm entry in another naked egg. The entrance cone formed as before, *A, B*. It engulfed

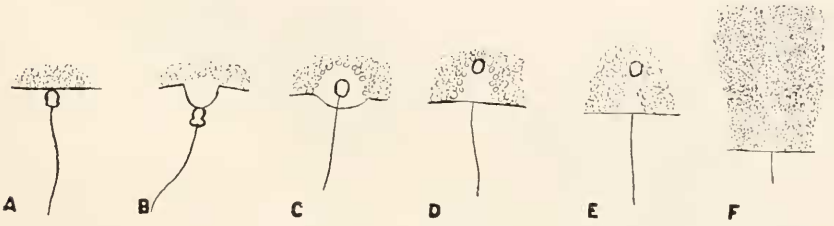

Fig. 20. Several steps in the successful insemination of a naked egg.

the sperm-head and then receded as the sperm-head rapidly moved inward along an ever-deepening hyaline pathway within the egg, *C, D* and *E*. The sperm-head produced a typical sperm-aster, *F*, and the egg segmented in a normal manner.

The striking features which are brought out in the behavior of the naked egg are as follows: First, the spermatozoön touches the sur-

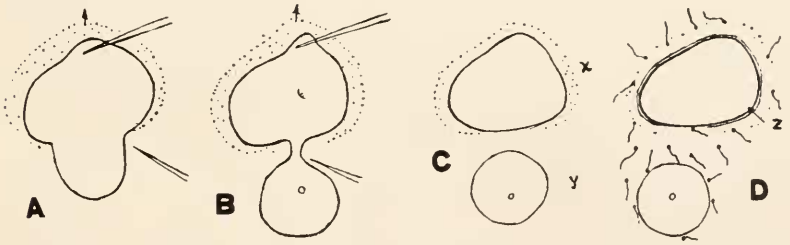

Fig. 21. Production of an endoplasmic exovate by cutting a gash in the cortex of an unfertilized egg and causing the interior to flow out. Ectoplasmic remnant, *x*, is fertilizable. Endoplasmic sphere, *y*, is unfertilizable. For description see text.

face of the egg before there is any evidence of a cone. Second, a cone forms after the sperm is in contact with the egg. Third, the cone forms no filamentous process such as is seen when a mass of jelly in-

tervenes between the cone and the spermatozoön. Fourth, no fertilization membrane whatever is produced.

The insemination of these naked eggs also bears on the question of the existence of a specific attraction of the egg to spermatozoa. Spermatozoa frequently swim up to a naked, unfertilized egg, wander along its surface and then swim away. Apparently the formation of an entrance cone is dependent on something more than the mere presence of a spermatozoön on its surface.

8. Insemination of Squashed Eggs

These experiments show the behavior, toward spermatozoa, of the egg-cortex as contrasted with that of the extruded interior.

Fig. 21 represents the artificial production of an endoplasmic exovate and the behavior of the isolated exovate and of the ectoplasmic remnant to insemination. A deep gash was first made with a needle in one side of an unfertilized, mature starfish egg. With a second needle the other side of the egg was seized and pulled to the shallow edge of a hanging drop, *A*. The interior of the egg flowed out at the spot where the gash was made. The fluid exovate rounded up as its connection with the more solid, cortical remnant of the egg became constricted. By gentle manipulation, *B*, the neck pinched off so that the egg was thus divided, *C*, into an ectoplasmic remnant still maintaining its jelly investment, *x*, and a naked endoplasmic sphere, *y*. As already described (Chambers, 1923), the endoplasmic spheres are unfertilizable. On the other hand, the ectoplasmic remnant is readily fertilizable and may develop into a swimming larva. The difference in behavior of the two pieces when inseminated is shown in *D*. The ectoplasmic remnant produced an entrance cone with its filament and the attached spermatozoön readily entered, *z*, in *D*, and was followed by the lifting of a typical, though collapsed, fertilization membrane. The endoplasmic sphere showed no reaction to the presence of the spermatozoa. Some hit it head on, others wandered over its surface, sometimes remaining motionless for a few seconds, only to swim away. No cones formed on the sphere and no spermatozoön was ever observed to enter. In a previous communication (Chambers, 1921) I stated that the endoplasmic spheres never segment, although I assumed that spermatozoa may enter. This assumption was based on the sections of several endoplasmic spheres which contained numerous small chromatic bodies which I took to be unaltered sperm-heads. In the light of more recent results I reexamined the slides containing these sections and found that the chromatic bodies are far too small to be sperm-heads; they also differ in being rod-shaped and are probably bacterial organisms. They certainly

are not spermatozoa. All the other endoplasmic spheres (eighteen in all) which were sectioned and stained showed no bodies even remotely resembling sperm-heads, although they had been heavily inseminated before fixing.

Fig. 22, *A* shows an egg which was torn and squashed. The original

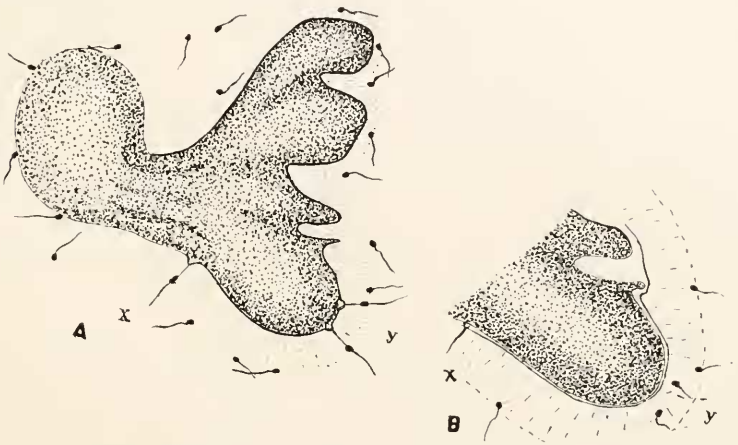

Fig. 22. Insemination of a torn and squashed egg. For description see text.

cortex was maintained on the part still covered by the jelly. Upon the addition of spermatozoa, cones formed on the original cortex at *x* and *y*. Two minutes later, *B*, the sperm at *x*, had entered while the two sperm at *y* were discarded. Note that the fertilization membrane formed only on the original cortex.

Discussion

Filamentous structures have been known to develop on the surface of Echinoderm ova. Some of them are delicate, wavy, cylindrical bodies which often appear when the eggs are placed under abnormal conditions of pressure, temperature, hypertonicity of their environment, etc. They are probably degeneration phenomena.

Other filamentous structures of quite a different sort have been noted on eggs after exposure to spermatozoa. Such are the structures described by Seifriz (1926) and Hobson (1927), which are identical with the flame-like processes which Fol long ago described as growing out from the " cones d'exudation " at the site of sperm-penetration. In immature eggs these flame-like processes attain considerable lengths. They slowly change in shape and size although Seifriz found them to be extraordinarily stiff when manipulated with microneedles. The in-

semination filaments described in this paper resemble the flame-like processes of the exudation cones except that they are extremely tenuous and usually are single instead of multiple. They also possess a stiffness which is quite at variance with the limp, filamentous outgrowths on degenerating eggs.

The results described in this paper indicate that the insemination filaments of mature eggs develop with extraordinary rapidity, but when they retract the process is a gradual one. Because of this, it has been impossible to determine directly whether the insemination filament is an outgrowth from the sperm-head to the cone or whether it emanates from the cone itself. Fol argues against the former possibility, because there is no apparent decrease in volume of the sperm-head. Another case in point is the relatively weak attachment of the filament to the sperm-head, for, whenever the filament is broken, either mechanically or spontaneously (e.g., in the case of incomplete polyspermy), the separation occurs at the head of the sperm and not at the cone. The main argument in favor of the filament being an outgrowth of the entrance cone is that it has been actually observed to develop from the cone in immature and in old, mature eggs in which all the other steps of the insemination process are identical with those of freshly matured eggs. Occasionally an abortive filament has been observed to arise from a cone without encountering a spermatozoön, and later to recede.

The development of the typical insemination filament appears to be a peculiar adaptation to the presence of the radially structured jelly about the eggs, because, when the jelly is completely removed, no filaments develop and insemination occurs by the elevation of an ovoid cone which engulfs the spermatozoön.

An extraordinary feature in the insemination process of the starfish egg is the apparently passive rôle which the spermatozoön plays in its migration through the jelly to the entrance cone. All the evidence indicates that the movement of the spermatozoön is due to the progressive shortening of the insemination filament. In this regard it is significant that occasionally the connection of the filament with the head of the spermatozoön is at such an angle that the spermatozoön moves as if it were actually being dragged backward to the cone. The spermatozoön in such a position could hardly be moving under its own motive power.

The main evidence for concluding that the insemination process described in this paper is normal, is the fact that the fertilization membrane always first rises over the cone at the base of the filament to which the approaching spermatozoön is attached and its elevation then spreads progressively from this site over the entire surface of the egg.

In the presence of too many sperm an egg frequently responds by

developing more than one filament with the result that several spermatozoa begin to migrate through the jelly. As the eggs age there is an increased production of filaments. The successful penetration into the egg of one spermatozoön and the failure of another to do so is conditioned by a definite time relation. It is possible for all of several spermatozoa to penetrate the egg if they begin migrating through the jelly within a few seconds of one another. Their success in entering the egg bears no relation to their distance from one another on the surface of the egg but to the time when the filaments begin to draw them in. In freshly matured eggs polyspermy tends to be prevented because of the paucity of insemination filaments. If, out of several migrating inward, one spermatozoön is sufficiently ahead of the others, polyspermy may be prevented by a gradual attenuation of the delayed filaments which finally break loose from the spermatozoa attached to them. Sometimes a delayed filament does not lose its spermatozoön, but continues retracting until the spermatozoön arrives on the cone. The spermatozoön, however, fails to enter the cone because of the elevating fertilization membrane which has already begun to spread from the region of another more successfully functioning cone. In such a case the spermatozoön is definitely discarded by a peculiar process which Just evidently saw when he described a spermatozoön being "pushed off from the egg, a delicate strand connecting the tip with the apex of the cone."

Just (1929) claims that filaments which are formed as a response to insemination occur only on abnormal ova and are exaggerated entrance cones. The only observation which he records of a strand connecting the sperm with the cone is one which he states occurred when the sperm was "pushed off from the egg." Such a case I have also frequently observed on abnormal eggs. My crucial observations of the true insemination filament were on fresh maturing eggs, from lots of which over 95 per cent segmented and developed normally. Fixed material is not suitable for a study of the movement of spermatozoa to the surface of the egg. Our difference of opinion on living eggs is one of observation, the methods we both used being presumably the same.

Quoting from Just, the "spermatozoa rush toward the jelly hull; of these, one, rapidly moving through it, reached the egg within 5 seconds." Although this rapidity of the movement is greater than any which I have observed, it is to be noted that Just admits the passage, through the jelly, of only one out of many; the others remain outside.

I have shown the phenomenon to several competent cytologists at Woods Hole during the past summer. They agreed with me in the

observation that the one migrating spermatozoön, during its passage through the jelly to the egg, is connected by an ever-shortening, straight filament to the entrance cone into which the head of the sperm finally disappeared. Moreover, the elevation of the fertilization membrane was observed to start over the base of this particular cone.

Summary

1. Evidence is given to indicate that the formation of insemination filaments is the normal procedure of fecundation in the starfish egg. These filaments extend from the egg's surface to the spermatozoa lying on the outer borders of the jelly surrounding the egg.

2. The spermatozoön on the end of an insemination filament moves to the egg through the jelly by no apparent motive power of its own. This movement is accompanied by a progressive shortening and thickening of the filament.

3. The fertilization membrane begins to rise off the cone by the time the spermatozoön has migrated about halfway through the jelly. The elevation of the membrane spreads from this region.

4. The filament is a peculiar adaptation to the presence of the relatively dense jelly surrounding the egg and to the inability of the blunt-headed spermatozoa to reach the egg. In the absence of the jelly only an ovoid entrance cone develops to receive the spermatozoön.

5. Polyspermy can be prevented by the breaking loose of supernumerary insemination filaments from their attached spermatozoa. The discarded spermatozoa remain motionless in the jelly while the filaments are completely withdrawn.

6. There is a definite relation between the time that two or more spermatozoa become attached to insemination filaments and the success of one or all to enter the egg. This bears no relation to the distance of their places of attachment on the surface of the egg but to the time when the filaments begin to retract.

7. The original cortex is the only part of the starfish egg which responds to insemination. Endoplasmic exovates do not become inseminated.

BIBLIOGRAPHY

Chambers, R., 1923. Studies on the Organization of the Starfish Egg. *Jour. Gen. Physiol.*, **4**: 41.

Lillie, F. R., and Just, E. E., 1924. Fertilization, Section VIII in General Cytology. University of Chicago Press.

Just, E. E., 1929. The Production of Filaments by Echinoderm Ova as a Response to Insemination, with Special Reference to the Phenomenon as Exhibited by Ova of the Genus Asterias. *Biol. Bull.*, **57**: 311.

Fol, H., 1877. Sur le premier développement d'une Étoile de mer. *Compt. Rend. Acad. Sci.*, **84**: 357.

Fol, H., 1879. Recherches sur la fécondation et la commencement de l'hénogénie chez divers animaux. *Mém. de la Soc. de phys. et d'hist. nat. de Genève,* **26**: 89.

Chambers, R., 1921. Microdissection Studies, III. Some problems in the maturation and fertilization of the Echinoderm Egg. *Biol. Bull.,* **41**: 318.

Seifriz, W., 1926. Protoplasmic Papillæ of *Echinarachnius* Oöcytes. *Protoplasma,* **1**: 1.

Hobson, A. D., 1927. A Study of the Fertilization Membrane in the Echinoderms. *Proc. Roy. Soc. Edin.,* **47**: 94.